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Hedgehog Signaling: An Arrestin Connection?

Arrestins are best known for terminating signaling by G protein coupled receptors. New binding, localization and genetic studies suggest that Arrestins may also participate in the transduction of Hedgehog signals by the seven transmembrane domain protein, Smoothened.

Daniel Kalderon

The Hedgehog (Hh) protein and its relatives mediate cell–cell communication in a wide variety of developmental contexts. Important details are still being learned about the mechanism by which the Hh signal is transduced in receiving cells. This is well illustrated by two recent papers [1,2] which report an unexpected connection between the signal transduction mechanisms used by Hedgehog (Hh) proteins and by G protein coupled receptors. Smoothened (Smo), the key cell-surface transducer of Hh signals, shares a seven transmembrane domain topology with G protein coupled receptors and has therefore long been suspected to signal by coupling to trimeric G-proteins [3]. Although this suspicion has neither been proven nor dismissed, it now seems possible that another aspect of G protein coupled receptor biochemistry, namely phosphorylation-dependent binding to Arrestin, may be fundamental to Smo activity.

Arrestin binding to prototypical G protein coupled receptors, such as rhodopsin or β2 adrenergic receptors, is dependent on both an agonist dependent change in G protein coupled receptor conformation and phosphorylation of the receptor on multiple serine or threonine residues by a G protein coupled receptor kinase (GRK) [4]. Arrestin binding can have several consequences. Most importantly it blocks signaling by impeding access of G-proteins to the activated receptor. Arrestin binding also induces clathrin-mediated receptor internalization, leading either to receptor dephosphorylation and recycling or to receptor destruction in lysosomes. Finally, G protein coupled receptor-bound Arrestin can recruit additional proteins to membranes, thereby initiating additional signal transduction pathways, as shown most clearly for mitogen activated protein (MAP) kinase pathways.

Very little can be said with certainty about the biochemistry of Smo in the Hh signaling pathway (Figure 1). Smo is not activated directly by Hh [3]. Rather, Smo is inhibited indirectly by Patched (Ptc), and this inhibition is relieved by binding of Hh to Ptc. Ptc is a 12 transmembrane domain protein, related both to bacterial proteins that transport small molecules across membranes and to molecules that regulate vesicle traffic [5]. Smo can be inhibited or activated by specific small cholesterol based compounds [6], and segregates into Ptc-free endosomes in response to Hh, thereby escaping lysosomal degradation [7]. Hence, it is conjectured that Smo activity is regulated by small intracellular ligands, its subcellular distribution or both.

Evidence for G-proteins as Smo effectors comes from studies showing an ability of Smo to couple to G_i in a heterologous system and by the production of a subset of Sonic hedgehog (Shh) loss-of-function phenotypes in zebrafish by pertussis toxin inhibition of G_i [3]. However, genetic modification of G protein activities, tested most extensively in *Drosophila* tissue culture cells, has not so far been found to alter Hh signaling [8]. The physical interaction of Smo with Cubitus interruptus (Ci), the transcriptional effector of the Hh pathway, via the kinesin-related protein Costal 2 provides a plausible alternative to G protein mediated signaling in *Drosophila* [9]. Furthermore, a

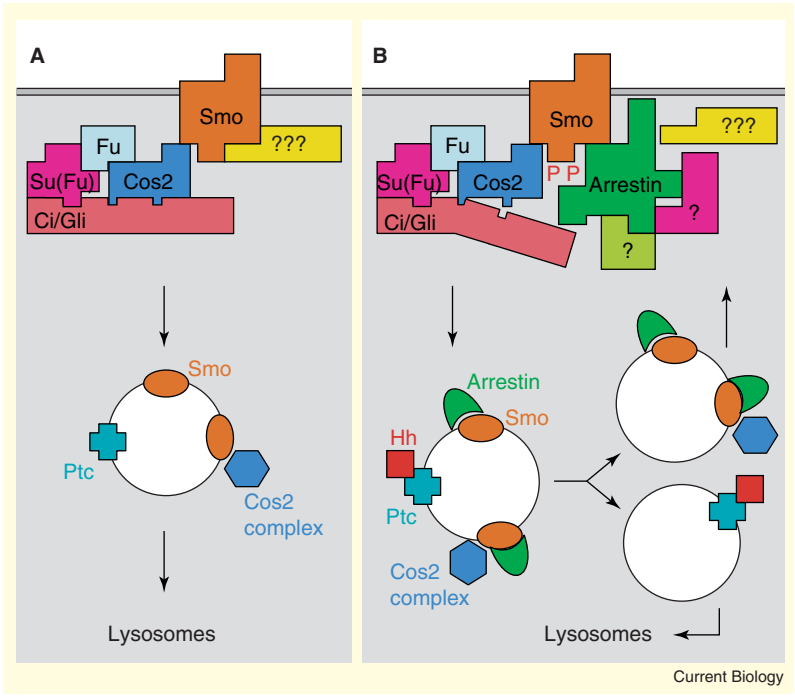

Figure 1. Possible roles of Arrestin in Hg signaling.

(A) In the absence of Hh, Smo and Ptc are endocytosed and degraded in lysosomes. Smo binds to Cos2 but Ci is effectively anchored in the cytoplasm. (B) Hh binds to Ptc, somehow causing segregation of Smo into Ptc-free vesicles and increased Smo phosphorylation. Binding of Arrestin to phosphorylated Smo could have a number of possible effects: it might promote recycling of Smo; alter contacts among Cos2 complex components to facilitate Ci/Gli release; recruit Arrestin binding proteins that act on the Ci–Gli–Cos2 complex; or prevent Smo from interacting with a hypothetical binding partner that would otherwise divert Smo from the Hh signaling pathway.

likely vertebrate version of Costal 2 has recently been characterized in zebrafish as acting in Hh signaling [10]. However, *Drosophila* Smo binds Costal 2 even in the absence of Hh, so an inducible biochemical change still needs to be identified to explain how the presence of Hh is transmitted to Ci by Smo.

Chen *et al.* [2] now report a set of investigations involving transfected mammalian tissue culture cells that demonstrate recruitment of β-Arrestin2 to the plasma membrane by Smo. This is inhibited by Ptc and by the Smo anatagonist cyclopamine, and accentuated by Shh (in the presence of Ptc) and by cholesterol-based Smo agonists. Furthermore, phosphorylation of vertebrate Smo was found to be dependent on GRK2 activity, to be inhibited by Ptc and cyclopamine and to be increased by Smo agonists. These observations of activity dependent phosphorylation and Arrestin binding directly mirror the properties of G protein coupled receptors such as the β2-adrenergic receptor.

What are the functional consequences of Arrestin's association with Smo? Surprisingly, Wilbanks *et al.* [1] found that morpholino depletion of β-Arrestin2 in zebrafish embryos produced phenotypes akin to those resulting from partial loss-of-function Smo mutations [1]. It will be important to see if these results can be confirmed and extended with classical loss-of-function mutations, in particular to determine if Arrestin is absolutely essential for Hh signal transduction. Neither β-Arrestin1 nor β-Arrestin2 knockout mice have significant developmental abnormalities [4], but some G protein coupled receptors can be desensitized by either β-Arrestin isoform, and both β-Arrestin1/2 double knockout mice and GRK2 knockout mice die as embryos. It has not been reported whether these embryos have altered Hh signaling.

Does Smo activity in *Drosophila* require Arrestin binding or signal-dependent Smo phosphorylation? While neither Arrestin nor GRK has yet been implicated in *Drosophila* Hh signaling, such roles have not yet been excluded by genetic investigations. The hypothesis that Smo phosphorylation is required for activity was actually first formulated for *Drosophila*, based on an observed increase of Smo phosphorylation in response to Hh [11], and this has recently been extended by the identification of specific phosphorylation sites essential for Smo activity [12–14]. These sites are not, however, GRK sites but protein kinase A (PKA) and casein kinase 1 (CK1) sites, and they are not conserved in vertebrate Smo. Might the PKA and CK1 sites of *Drosophila* Smo serve the same purpose of binding Arrestin as proposed for GRK2 sites in vertebrate Smo?

Vertebrate β-Arrestins can each bind many different G protein coupled receptors and do not exhibit marked selectivity for specific phosphopeptide sequences [15]. Rather, it seems that Arrestin binding is progressively tighter as more phosphorylated residues are presented, and the preference of GRKs for locally acidic substrates facilitates the generation of highly phosphorylated receptors [4]. The clustered PKA, PKA-primed CK1 sites and CK1-primed CK1 sites of *Drosophila* Smo would also be expected on theoretical grounds to be cooperatively and extensively phosphorylated. There is some evidence that these sites need to be extensively phosphorylated to contribute to Smo activity: Smo was inactivated only by alterations predicted to eliminate multiple PKA and CK1 site phosphorylations, and Smo was constitutively activated only by replacing multiple such sites with acidic residues [13,14]. So it is conceivable that PKA and CK1 sites in *Drosophila* serve to bind Arrestin.

Drosophila Smo might also have GRK phosphorylation sites. *Drosophila* Smo, isolated from Hh-responding cells, was found to have no less than 26 distinct

phosphorylated residues, of which 11 are accounted for by consensus PKA and CK1 sites [14]. The remaining sites occur in clusters and most are in the vicinity of acidic residues, as found for GRK sites in the β2-adrenergic receptor. It remains to be determined whether the increased phosphorylation of Smo accompanying Hh signaling can be attributed to PKA, CK1, GRK or other protein kinase sites in Smo.

If Arrestin binding to phosphorylated Smo plays an important positive role during Hh signaling, what might that role be? The answer seems likely to involve the regulation of Smo subcellular localization. The evidence to date is that agonist-stimulated internalization of vertebrate Smo, accompanied by β-Arrestin2, is dependent on GRK2 and β-Arrestin2, but the fate of internalized Smo and Arrestin is not clear [2]. For *Drosophila* Smo, assayed in tissue culture cells with overexpressed proteins, loss of PKA and CK1 sites reduces Hh-induced surface localization, while conversion of those sites to acidic residues increases Smo surface localization even in the absence of Hh [13]. So, PKA and CK1 phosphorylation of Smo appears to either decrease Smo endocytosis or enhance recycling of Smo to the plasma membrane. It thus remains to be determined whether GRK2 and PKA/CK1 phosphorylation affect the same or different steps in the Smo internalization and recycling that normally accompanies Hh pathway activation.

It is not clear whether Hh-dependent changes in Smo subcellular localization or Arrestin binding are part of the activation mechanism or effector mechanism for Smo. As GRK phosphorylation and Arrestin binding require that Smo be activated in some way and likely lead to altered Smo subcellular distribution [2], a role for Arrestin in the acute activation of Smo seems unlikely. But Hh signaling, unlike the activation of most G protein coupled receptors, must be maintained over long time periods, so it is possible that Arrestin allows continued Smo activation by recycling Smo proteins that would otherwise become desensitized or directed to lysosomes for destruction. Alternatively (or additionally), Arrestin may be a Smo effector. Perhaps Smo-bound Arrestin facilitates release of Ci (or Gli proteins) from cytoplasmic complexing partners. This might be achieved by altering binding interactions among known complexing proteins or by recruiting novel effectors (such as phosphatases or a cAMP phosphodiesterase) to the complex either through direct binding to Arrestin or by altering the subcellular location of the Ci/Gli complex.

Even though mechanistic conservation between vertebrate and *Drosophila* Smo cannot be safely predicted, we might be informed by looking even further afield to Frizzled (Fz) proteins, the closest known relatives of Smo. Fz proteins transduce extracellular Wnt signals into transcriptional responses through a β-catenin dependent pathway but also initiate distinct 'non-canonical' pathways affecting cell shape or polarity and using different signaling intermediates [16]. As for Smo, the issue of whether Fz couples to G-proteins has been hard to resolve. Strong arguments have been made, using native Fz proteins or chimeras of Fz and β-adrenergic receptors, that Fz proteins can activate G proteins and require G protein activity to activate a non-canonical Fz pathway involving changes in Ca^{2+} [17]. In studies similar to those linking Smo and Arrestin, it was found that internalization of Fz4 in response to Wnt5A and PKC activation requires β-Arrestin 2 [4]. Here Dishevelled, an essential component of all Fz pathways, likely acts in a phosphorylated form as a bridge between Arrestin and Fz. The functional consequences of Arrestin-dependent internalization of Fz proteins have not, however, been described.

Recent genetic studies in *Drosophila* [18] provide evidence that a specific G protein, G_0, is a Fz effector in both the canonical β-catenin pathway and the Fz planar cell polarity pathway. While these studies do not reveal the precise biochemical role of G_0 or prove that Fz signaling is channeled exclusively through G_0, they certainly provoke speculation about which Fz paradigms are shared by Smo.

More extensive testing might reveal at least a small positive role for G proteins in relaying a Smo signal. Activation of a G protein by Smo could also explain how Smo becomes phosphorylated by a GRK, as GRKs are normally brought to G protein coupled receptors in part by binding to free Gβγ subunits.

A more convoluted speculation comes from evidence that activation of one Fz signaling pathway at the expense of another can be regulated by Fz subcellular localization or by the status of Disheveled phosphorylation [19,20]. Perhaps Smo too can engage in molecular interactions, maybe even with G-proteins, that can divert its activity from the Hh signal transduction pathway. In this case binding of Arrestin to phosphorylated Smo might directly block a diversionary interaction of Smo or might localize Smo to a specific cellular site where it can only engage in molecular interactions that activate Hh signal transduction.

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Mitosis: PARty Time in the Spindle

Poly(ADP-ribose), a post-translational protein modification known to affect chromatin structure, has now been shown to regulate microtubule organization during mitosis. These findings alter conventional views of the mechanisms of spindle assembly and function.

Duane A. Compton

Accurate chromosome segregation is essential for cell viability and is performed by a complex structure called the spindle. Spindles are primarily composed of microtubules, and chromosome movement relies on the dynamic properties of these polymers. The conventional view is that the organization of microtubules into a symmetric bipolar spindle is driven by microtubule-associated proteins — motor and non-motor crosslinking proteins — that are activated by phosphorylation at the proper time during the cell cycle [1]. New data [2] have revealed that poly(ADP-ribose), or PAR, is required for assembly and maintenance of bipolar spindles. These findings indicate that modification by poly(ADP-ribose) — PARsylation — is a way of regulating the activity of spindle proteins, and raise the possibility that PAR might have a mechanical function in spindles.

PAR was identified in nuclear extracts from cells more than 40 years ago [3]. PAR is rapidly induced at sites of DNA damage, where it influences chromatin structure when affixed to histones [4]. Recently, PAR has also been implicated in the regulation of tissue-specific gene expression by influencing chromatin structure in a manner akin to acetylation [5]. PAR is synthesized by enzymes known as poly(ADP-ribose) polymerases, or PARPs. There are as many as 18 genes for PARP-related proteins in mammals. PARPs transfer ADP-ribose from the cofactor NAD+ to glutamic acid residues on specific target proteins (Figure 1A); they also catalyze the formation of long, branching polymers of ADP-ribose, via ribosyl-ribose glycosidic linkages, through processive enzymatic activity [6]. PAR levels in cells are typically very low as PARP activity is antagonized by the enzyme poly(ADP-ribose) glycohydrolase (PARG), which releases ADP-ribose from both PAR and PARsylated proteins (Figure 1A) [7].

Chang *et al.* [2] hypothesized that PAR might regulate spindle assembly, on the basis of anecdotal evidence that various PARPs localize to spindles or associate with spindle proteins [4,6,8]. Consistent with this hypothesis, PAR and PARG both localize to spindles in frog egg extracts and mammalian tissue culture cells. PAR is enriched at spindle poles and centromere/kinetochores, but also throughout the body of the spindle, particularly in frog egg extracts (Figure 1B). Importantly, biochemical fractionation of frog egg extracts revealed significant enrichment of PARsylated proteins on spindles relative to the total extract.

To test PAR's suggested function in mitosis, Chang *et al.* [2] added excess PARG to frog extracts: the consequent reduction in PAR level did not alter spindle pole organization, but it inhibited both the formation and maintenance of bipolar spindles by disrupting the association between half-spindles. This effect would appear to be specific to PAR, because excess PARG is ineffective in the presence of a PARG-specific inhibitor, and the same effect is observed on addition of PAR-specific antibodies. PAR is thus critical for establishing and maintaining spindle bipolarity, perhaps through stabilizing anti-parallel microtubule interactions in the central spindle (Figure 1B).

Lastly, Chang *et al.* [2] measured PAR dynamics in spindles in frog egg extracts using fluorescently tagged Fab fragments of PAR-specific antibodies, at concentrations that do not perturb spindle bipolarity.